UNIVERSITI PUTRA MALAYSIA

ANTI-ANGIOGENIC POTENTIAL OF ARDISIA CRISPA ROOTS ETHANOLIC EXTRACT AND ITS QUINONE-RICH FRACTION IN MICE

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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July 2013

Chair: Roslida Abd Hamid @ Abd Razak, PhD

Faculty: Medicine and Health Sciences

Angiogenesis is the process of blood vessel formation which plays a crucial role in normal physiology, and also in the progression of various chronic diseases such as cancer, arthritis and such. As targeting angiogenesis has become an important strategy in the search of treatments of various debilitating ailments, it is a need-based study to identify a natural source of anti-angiogenic agent that may halt the progression of angiogenesis event. *Ardisia crispa*, locally known as "mata itik" (Family: Myrsinaceae) has been used in traditional Malay medicine to treat various ailments related to inflammation. *Ardisia crispa* roots have been shown to treat various inflammation-related diseases in several previous studies. As angiogenesis is strongly correlated with inflammation, the aim of the present study was to evaluate anti-angiogenic potential of the hexane partition of *Ardisia crispa* roots ethanolic extract (ACRH) and its quinone- rich fraction (QRF) on several experimental models, namely Miles vascular permeability test, murine air pouch granuloma and mouse sponge implantation test. Preliminary cyclooxygenase and soy lipoxygenase inhibitory study were also conducted to elucidate the possible pathways involved.

Preliminary phytochemical screening of ACRH indicated the abundant presence of flavonoid, triterpene and tannin. Quinone- rich fraction (QRF) was separated from ACRH (38.33% w/w) and further isolated to yield a compound, namely fAC-2, indicated by a single TLC spot at R_f: 0.76. The compound was later found to be impure, when later analysed with GC-MS. Nevertheless, fAC-2 was elucidated to possess a major constituent of a benzoquinonoid compound (2-methoxy-6-undecyl-1, 4-benzoquinone), when compared with the standard data. Both ACRH and QRF were also quantified using high performance liquid chromatography (HPLC). For toxicity study, the LD_{50} value of ACRH was found to be 617.02 mg/kg. In Miles vascular permeability assay, the lowest dose of both ACRH and QRF (10 mg/kg) produced significant reduction in VEGF-induced hyperpermeability compared to vehicle control. In murine air pouch granuloma, ACRH and QRF displayed significant and dose-dependent angiogenic and inflammatory inhibition, in which significant reduction of vascular index and granuloma tissue weight was observed at high dose (100 mg/kg). ACRH and QRF were also shown to possess selective COX-2 inhibitory properties which were dose-dependent, though COX-1 inhibition was also observed in a lower percentage. On the other hand, ACRH and QRF did not exhibit LOX inhibitory activity. Interestingly, fAC-2 showed its selectivity towards the inhibition of COX-2, instead of COX-1, and showed to be a moderate LOX inhibitor. Thus, it can be concluded that *Ardisia crispa* roots showed potential anti-angiogenic properties by partly mediating COX-2 activity, as shown in the *in vitro* screening, and it is postulated that fAC-2 (2-methoxy-6-undercyl-1, 4-benzoquinone) displays dual COX-2 and LOX once it is purely isolated in a large scale and tested *in vivo*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

POTENSI AKAR *Ardisia crispa* EKSTRAK ETANOL DAN FRAKSI KAYA KUINON SEBAGAI PERENCAT PEMBENTUKAN SALUR DARAH KE ATAS MENCIT

Oleh

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Pembentukan salur darah merupakan satu proses yang penting dalam fisiologi normal, dan juga penting dalam perkembangan pelbagai jenis penyakit kronik seperti kanser, radang otot, dan sebagainya. Oleh sebab pensasaran pembentukan salur darah semakin menjadi strategi yang penting dalam pencarian rawatan bagi pelbagai penyakit, adalah penting untuk kajian dijalankan bagi mengenal pasti sumber semulajadi bagi agen perencat pembentukan salur darah, yang mampu merencat perkembangan proses yang berlaku di dalam penghasilan salur darah. *Ardisia crispa* (Famili: Myrsinaceae) yang juga dikenali sebagai pokok mata itik, telah lama digunakan dalam perubatan tradisional Melayu untuk mengubati penyakit yang berkaitan dengan keradangan. Akar *Ardisia crispa* telah terbukti mampu merawat pelbagai penyakit yang berkaitan dengan keradangan melalui beberapa kajian yang dijalankan sebelum ini. Oleh sebab terdapat korelasi yang kuat antara keradangan dan pembentukan salur darah, objektif penyelidikan ini dilakukan adalah untuk mengetahui sama ada partisi heksana (ACRH) daripada ekstrak mentah akar pokok *Ardisia crispa,* beserta fraksi kaya kuinon (QRF) mempunyai aktiviti merencat

pembentukan salur darah dalam beberapa model eksperimen, termasuk ujian ketelapan vaskular Miles, granuloma kantung udara murin dan ujian implan span dalam mencit. Kajian awal terhadap penglibatan enzim siklooksigenase (COX) dan lipoksigenase (LOX) juga dijalankan untuk mengenalpasti mekanisma yang mungkin terlibat dalam perencatan formasi salur darah. Kajian awal terhadap komposisi fitokimia ACRH menunjukkan bahawa ACRH mengandungi sebatian-sebatian flavonoid, triterpena dan tannin yang banyak. Fraksi kaya kuinon (QRF) telah dipisahkan daripada ACRH (38.33% w/w) dan seterusnya diasingkan untuk menghasilkan satu sebatian utama (fAC-2) berdasarkan bintik tunggal pada plat Kromatografi Lapisan Nipis (KLN) pada R_f:0.76. Sebatian tersebut kemudiannya didapati tidak tulen melalui analisis Kromatografi Gas-Spektroskopi Jisim (KG-SJ). Walaubagaimanapun sebatian benzokuinon iaitu 2-metoksi-6-undesil-1,4-benzokuinon dibuktikan merupakan komponen utama dalam sebatian tersebut,(fAC-2) apabila dibandingkan dengan data piawai. ACRH dan QRF juga diukur dengan menggunakan kromatografi cecair prestasi tinggi (KCPT). Untuk kajian ketoksikan, nilai LD_{50} bagi ACRH telah dikenalpasti sebagai 617.02 mg/kg. Dalam ujian ketelapan vaskular Miles, dos terendah bagi ACRH and QRF (10 mg/kg) menghasilkan pengurangan yang signifikan dalam ketelapan hiper yang dirangsang oleh VEGF. Dalam granuloma kantung udara murin, ACRH and QRF menunjukkan kebergantungan dos yang signifikan dalam perencatan formasi salur darah dan keradangan, di mana pengurangan yang signifikan dalam indeks vaskular, dan juga berat kasar tisu granuloma juga dapat diamati. Aktiviti perencatan formasi saluran darah oleh ACRH and QRF telah terbukti disebabkan oleh enzim siklooksigenase-2 (COX-2) secara selektif, walaupun aktiviti enzim siklooksigenase-1 (COX-1) juga direncat, dalam peratusan yang lebih rendah. Walaubagaimanapun, sebatian fAC-2

telah terbukti menunjukkan sifatnya yang selektif terhadap perencatan aktiviti COX-2, berbanding COX-1. Sebatian fAC-2 juga menunjukkan perencatan yang baik terhadap enzim LOX soya. Kesimpulannya, akar *Ardisia crispa* menunjukkan potensi sebagai perencat formasi salur darah, dengan sebahagiannya melalui perencatan aktiviti enzim COX secara selektif, terbukti melalui ujian penyaringan *in vitro*. Walaubagaimanapun, sebatian fAC-2 berpotensi untuk menjadi agen perencat dwienzim COX-2 dan LOX setelah diasingkan dan ditulenkan melalui skala besar dan diuji secara *in vivo* pada masa yang akan datang.

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"A path without a heart is never enjoyable. On the other hand, a path with heart is easy— it does not make a warrior work at liking it; it makes for a joyful journey; as long as a man follows it, he is one with it."

Carlos Castaneda

I certify that a Thesis Examination Committee has met on 20 June 2013 to conduct the final examination of Siti Zaleha binti Raduan on her thesis entitled "Anti-Acute Inflammatory Effect of *Hibiscus rosa-sinensis* L. and *Hibiscus rosa-sinensis* var. *alba* Flower and Leaf Ethanol Extracts and Its Mechanism of Action in Rats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science

Members of the Thesis Examination Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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DAYANG ERNA ZULAIKHA AWANG HAMSIN

Date: 12 July 2013

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LIST OF ABBREVIATIONS

5-HETE	5-Hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxyeicosatetraenoic acid
5-LOX	5-lipoxygenase
ACR	*Ardisia crispa* roots
ACRH	*Ardisia crispa* roots hexane extract
ACUC	Animal Care and Use Committee
Ang1	Angiopoietin 1
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
COX-3	Cyclooxygenase-3
COXIB	Cyclooxygenase inhibitor
CFR	Code of Federal Regulation
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FAP	Familial Adenomatous Polyposis
FCA	Freund's Complete Adjuvant
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FLAP	5-Lipoxygenase Activating Protein
HGF	Hepatocyte growth factor
HPLC	High Performance Liquid Chromatography
IFN-α	Interferon- α
IFN-β	Interferon- β
IFN-γ	Interferon- γ
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
LAK	Lymphocyte-activated Killer
LIF	Leukemia inhibitory factor
LOX	Lipoxygenase
LSD	Least Significant Difference
LT	Leukotriene
LTA4	Leukotriene A_4
LTB4	Leukotriene B_4
LTC4	Leukotriene C_4
LTD4	Leukotriene D_4
MCP-1	Monocyte Chemotactic Protein-1

MMP	Matrix Metalloproteinase
MMP-2	Matrix Metalloproteinase-2
MMP-8	Matrix Metalloproteinase-8
MVD	Mean Vascular Density
NOS	Nitric Oxide Species
NRP-1	Neuropilin-1
NSAID	Non-Steroidal Anti-Inflammatory Drug
oxLDL	Oxidized Low Density Lipoprotein
p.o.	*per os* (orally)
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffer Saline
PDGF	Platelet-derived Growth Factor
PECAM	Platelet Endothelial Cell Adhesion Molecule
PGD2	Prostaglandin D_2
PGE2	Prostaglandin E_2
PGF2	Prostaglandin F_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG2	Prostaglandin G_2
PGI2	Prostaglandin I_2
pO2	partial Oxygen
QRF	Quinone Rich Fraction
R_f	Retention factor
R_t	Retention time
SEM	Standard Error of Mean
SPARC	Secreted Protein Acidic and Rich in Cysteine
TAE	Tris-acetate-EDTA
TGF-h	Transforming Growth Factor-h
TIMP	Tissue Inhibitor of Metalloproteinases
TLC	Thin Layer Chromatography
TMPD	Tetramethylphenylenediamine
TNF-α	Tumor Necrosis Factor- α
TXA2	Thromboxane-A_2
USC	United States Code
VE-cadherin	Vascular Endothelial-cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VEGI	Vascular Endothelial Growth Inhibitor
VI	Vascular Index
WHO	World Health Organization

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